Deamidation Kinetics of Casein Precipitated by Carbon Dioxide Compared with Commercial Caseinates

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———————— ABSTRACT ————————

Casein precipitated by carbon dioxide (CO_2-casein), and commercial sodium and calcium caseinates (1% w/v in water) were thermally deamidated at pH 8 for up to 96h at 85, 100, and 115°C. CO_2-casein displayed the highest overall extent of deamidation at 100 and 115°C after 32 and 24h, respectively, while there was no difference at 85°C. Structural differences between CO_2-casein and the commercial caseinates may account for the difference in deamidation. Deamidation kinetics were apparent first-order with respect to concentrations of the amides, asparagine and glutamine. Reaction rate constants and Arrhenius parameters were comparable from regression modeling.

Key Words: deamidation, casein, amides, kinetics, carbon dioxide

INTRODUCTION

DEAMIDATION OF A PROTEIN INVOLVES HYDROLYSIS OF THE AMIDE GROUP OF ASPARAGINE (Asn) or glutamine (Gln) to a carboxylic group, with release of ammonia. The reaction can be catalyzed by acid or base (Wright, 1991a). In a comparative analysis between soy protein and lysozyme, Zhang et al. (1993a) reported that deamidation rate was more sensitive to protein structure and specific amide content at basic conditions than at acidic conditions. Thus, deamidation under basic conditions would be useful to compare proteins.

Different reaction mechanisms occur at acidic and basic conditions. Reaction under acidic conditions is relatively rapid for both Asn and Gln and involves direct hydrolysis of the amide residue on the protein (Shih, 1992). However, reaction at basic conditions leads to formation of an intramolecular cyclic imide intermediate between a peptide nitrogen on the protein backbone and the carbonyl group of the amide of the Asn or Gln residue (Stephenson and Clarke, 1989; Tyler-Cross and Schirch, 1991).

For Asn, the reaction would involve a 5-membered succinimide ring
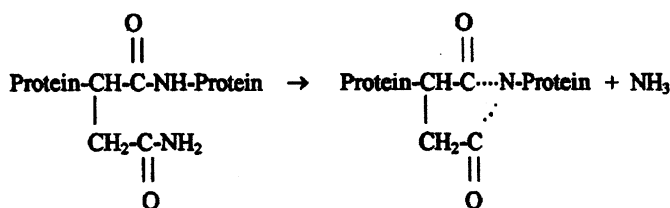
Analogously, Gln with its additional methylene group, would undergo a 6-membered imide ring formation.
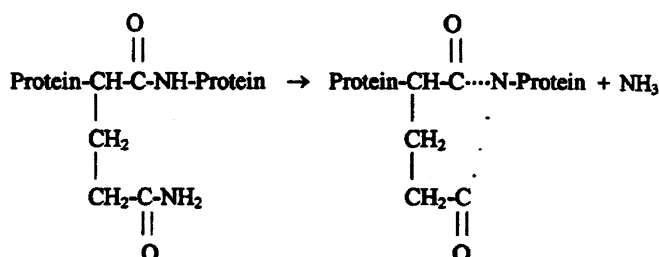
Due to steric hindrances caused by the extra methylene group, Gln deamidation would occur at a slower rate compared to Asn under basic conditions (Robinson et al., 1973; Wright, 1991b).

Deamidation changes the electrostatic charge on the protein residue from positive to negative by replacing an amide with a carboxyl group. Chemical modification has been a means of improving the functional properties of food proteins, which may improve and expand their use in the food industry. Ma and Khanzada (1987) thermally deamidated oat protein isolates under mild acidic conditions to increase their solubility and improve their emulsification and gelation. Friedl and Howell (1996) reported that thermally induced deamidation of gluten enabled it to more readily disperse in aqueous solutions. Egg white protein was deamidated with dry heat under alkaline conditions (Mine, 1997). Extents of deamidation as low as 12% improved gelation. Deamidation of other food proteins has been reported including soy (Matsudomi et al., 1985; Shih, 1991; Zhang et al., 1993a, b; Wagner and Guegen, 1995), zein (Casella and Whitaker, 1990), and sunflower (Claughton and Pearce, 1989).

With respect to casein, Zhang et al.(1993c) reported thermal treatment in low water environments produced deamidation of up to 60% of the total amide residues. Metwalli and van Boekel (1998) investigated the kinetics of heat-induced deamidation of sodium caseinate and reported a first-order dependence with respect to the total amide concentration during a reaction time of 2h. Enzymatic studies by Motoki et al. (1986) deamidated over 80% of the glutaminyl residues of α_{s1}-casein. They reported the aqueous solubility of the deamidated product showed a slight increase at acidic pH, and an increase of more than 2-fold in the presence of Ca^{2+}. Hamada (1992) reported partial protein hydrolysis prior to enzymatic treatment increased deamidation of casein amide residues by greater than 48%.

Production of CO_2-casein involves the acid precipitation of casein by dissolution of CO_2 in milk (Tomasula et al., 1995). Compared with mineral acids traditionally used in the production of commercial caseins, the use of CO_2 as a precipitant would have advantages because the resulting process is environmentally benign and the precipitant is eliminated from the products.

Our objective was to use deamidation to compare CO_2-casein to commercial sodium and calcium caseinates. Specifically, overall extent of deamidation as well as kinetics of the deamidation reaction was investigated. Additionally, the kinetic data may be useful to predict deamidation extent for deliberate chemical modification of caseins.

MATERIALS & METHODS

Materials

Sodium caseinate was purchased from Sigma Chemical Co. (St. Louis, MO). Calcium caseinate, Alanate™ 310, was purchased from New Zealand Milk Products (Santa Rosa, CA). CO_2-casein was prepared according to the method of Tomasula et al. (1995). Ammonium chloride standard solution (0.1M) and ionic strength adjustment solution (ISA) for electrode ammonia determination were obtained from Orion Research, Inc. (Boston, MA). ISA consisted of 5M NaOH/ 0.05M disodium EDTA/10% methanol with color indicator. ISA addition ensured a sample pH in the operating range of the electrode, which was pH>11. Trichloroacetic acid (TCA), from Sigma, was used as a protein precipitant to facilitate ammonia analysis. Distilled, de-ionized water was used for all reaction mixtures as well as for all ammonia determinations.

Conditions for kinetic studies

The method of Zhang et al. (1993b) was generally followed for the kinetic studies. Deamidation of each casein solution was performed at pH 8±0.002, at temperatures of 85, 100, 115 ±0.1°C; and sampling times were 2, 4, 6, 8, 24, 32, 48, 72, 96h with a control sampled at 0h. Aliquots (13 mL) of aqueous casein (1% w/v) were transferred to glass tubes with a rubber-lined black phenolic screw-cap (Fisher Scientific, Pittsburgh, PA). Additionally, a Kimble™ flat disc septum with PTFE-faced silicone rubber (Fisher Scientific) was securely placed into the cap to prevent ammonia vapor loss. The tightly capped vials containing the reaction mixture were placed in a convection oven (Despatch Industries, Minneapolis, MN) preset at a given constant temperature. The tubes were regularly shaken to ensure uniform reaction of the protein solution. Vial samples were withdrawn at specific time intervals and the reaction was quenched by immersing the vials in crushed ice (Zhang et al., 1993a; Sohn and Ho, 1995). The vials were then stored at 0°C until ammonia analysis was conducted which was no later than 2h after quenching.

Ammonia determination, C_{NH3}

Protein deamidation was assessed by measuring the ammonia liberated during the reaction. From reaction stoichiometry, 1 mol of ammonia molecule was produced per mol of amide group deamidated. Ammonia concentration, C_{NH3} (mmol/g protein), was determined using an ammonia ion-selective electrode (Orion Research, Inc., Boston, MA) as follows: An 8.5-mL aliquot of the reaction mixture was transferred to a centrifuge tube that contained 1.5 mL of 10% w/v aqueous TCA solution. This precipitated any soluble protein while leaving ammonia dissolved in the supernatant. The mixture was then centrifuged and the supernatant was diluted with 86 mL water and 4 mL ISA prior to using the ammonia ion-selective electrode. For each measurement, a calibration curve was prepared using standard ammonium chloride solutions ($1 \times 10^{-4} - 3 \times 10^{-4}$ M). Experiments were performed in triplicate.

Analysis of total protein amide content, C_{Ao}

Complete protein deamidation was performed to determine the total amide content of each casein, C_{Ao} (mmol/g protein)(Shih, 1990). A 5% w/v solution of casein in 2N HCl was heated under reflux for 3h. The total ammonia released was then measured by the electrode method. Measurements were made in duplicate. For kinetic calculations, initial values for concentrations (mmol/g protein) of Asn $(C_{Asn})_0$, and Gln $(C_{Gln})_0$, were based on standard bovine casein composition (Eigel et al., 1984) and assumed to be 31.4 and 68.6%, respectively, of the total casein nonpeptide amide content, C_{Ao}.

Deamidation calculations

$$C_A = C_{Ao} - C_{NH3}$$

C_A (mmol/g casein) was the concentration of the total remaining amide groups, i.e., the amide groups that were not deamidated. C_{Ao}

and C_{NH3} were determined as described. The fraction of total amide that was deamidated; i.e., deamidation percentage or extent of deamidation, was calculated as follows

$$\text{extent of deamidation (\%)} = [C_{NH3}/C_{Ao}] \times 100$$

Compositional analysis

Moisture, fat, ash , protein, and calcium contents were determined as described by Tomasula et al. (1995).

Statistical analysis

One-way analysis of variance was carried out using the F-test (Snedecor and Cochran, 1989) with values of $p<0.05$ indicating statistically significant differences.

RESULTS & DISCUSSION

TOTAL AMIDE CONCENTRATIONS, C_{Ao}, WERE 1.046, 1.044, AND 1.035 (mmol)/(g casein) for CO_2-casein, calcium caseinate and sodium caseinate, respectively. Based on these values, extent of deamidation was calculated (Table 1). Deamidation was fairly sensitive to temperature for each of the three caseins. Generally, a larger increase in extent of deamidation occurred from 85 to 100°C than from 100 to 115°C. Overall, increases in deamidation were (2.5- to 3-fold from 85 to 115°C as reaction time approached 96 h. Deamidation of sodium caseinate had been reported by Metwalli and van Boekel (1998) to show strong temperature sensitivity. The extent of deamidation increased 5-fold from 110 to 140°C at a heating time of 90 min.

Also, differences in extents of deamidation between CO_2-casein and the caseinates were more distinct (Table 1) as temperature and time progressed. At 85°C, extents of deamidation of CO_2-casein were not different than either of the commercial caseinates throughout the course of the reaction. At 100°C, CO_2-casein had deamidated more than the caseinates (p<0.05), but only after reaction time reached 32h. However, at 115°C, CO_2-casein started to deamidate more than the caseinates at reaction times of 24h and higher. Low extents of deamidation, i.e., relatively short heating times have been sufficient to induce sharp changes in the functional properties of proteins (Matsudomi et al., 1985; Hamada and Marshall, 1992). Thus, CO_2-casein should deamidate after thermal treatments similarly to commercial caseinates in practical applications.

At longer reaction times, structural differences between CO_2-casein and conventionally produced caseinates be important in the slightly greater deamidation of CO_2-casein. Compositional analysis (Table 2) revealed a similar make-up for all caseins. However, there was some difference with respect to calcium. Sodium caseinate had no detectable calcium, while the calcium in CO_2-casein was in a different form than that in calcium caseinate. Commercial casein is precipitated from milk by adjusting pH to 4.6 and heating to 35–40°C (Roeper, 1977). Under such conditions, the precipitation is brought about by solubilization of the colloidal calcium phosphate which disrupts the micelle structure and maximizes electrostatic interactions between individual casein micelles, which facilitates coagulation (Kinsella, 1984). Subsequently, the acid casein is neutralized with either sodium or calcium hydroxide. As a result, the calcium content of calcium caseinate exists in ionic form (Kinsella, 1984). CO_2-casein is precipitated at ≈pH 5.4. Casein precipitated at this pH may have a partially intact micelle structure (Tomasula et al., 1995). Thus, CO_2-casein could differ because it has some micellar structure and the calcium exists more in colloidal than in ionic form. This resulting structural difference between CO_2-casein and the caseinates possibly contributed to observed differences in extent of deamidation. Amino acids in close proximity to Asn or Gln through three-dimensional structure may catalyze deamidation through steric and chemical factors (Stephenson and Clarke, 1989; Wright, 1991b). For example, Asn deamidated relatively rapidly when followed by glycine or serine in peptide models (Geiger and Clarke, 1987; Tyler-Cross and Schirch, 1991; Wright, 1991b). Scotchler and Robinson (1974) reported that Gln preceded by arginine deamidated

Table 1—Effects of temperature and time on the percentage of amide groups that were deamidated for the caseins

	85°C			100°C			115°C		
Time	CO_2-cas.[a]	Ca-cas.[b]	Na-cas.[c]	CO_2-cas.[a]	Ca-cas.[b]	Na-cas.[c]	CO_2-cas.[a]	Ca-cas.[b]	Na-cas.[c]
(h)	(%)								
2	0.48	1.01	0.79	1.72	2.91	1.02	5.90	5.36	7.80
4	1.56	2.88	1.53	5.77	5.92	5.03	2.8	10.0	9.83
6	2.68	3.58	2.19	9.20	7.45	7.44	16.5	14.2	13.0
8	3.64	5.12	3.08	11.9	12.3	10.2	20.2	16.2	16.4
4	8.93	9.51	7.80	24.7	21.2	19.6	36.4	29.1*	31.9*
32	10.8	11.2	9.40	28.8	24.4	21.8*	41.8	33.8*	33.2*
48	13.4	16.7	12.0	34.6	28.7*	26.1*	52.0	40.0*	43.0*
72	18.5	21.4	15.9	37.4	28.8*	29.3*	58.8	48.2*	46.3*
96	21.6	23.4	18.8	40.2	34.0*	34.5*	64.4	55.6*	56.5*

[a]CO_2-cas. = CO_2-casein.
[b]Ca-cas. = calcium caseinate.
[c]Na-cas. = sodium caseinate.
*For particular caseinate, mean is significantly different (p<0.05) than that of CO_2-casein at given time and temperature.

Table 2—Compositional analysis of casein on a moisture-free, weight % basis

Casein	Protein	Ash	Fat	Ca^{2+}
Ca-cas.	96.4	3.74	0.93	1.65
Na-cas.	95.8	3.38	1.06	—[a]
CO_2-cas.	93.8	3.99	1.23	1.44

[a]None detected.

Table 3—Deamidation rate constants (⁻10³) of individual casein amides

	Asn			Gln		
	85°C	100°C	115°C	85°C	100°C	115°C
Ca-cas.	15.3	47.9	65.5	0.0038	0.0143	0.401
Na-cas.	10.1	37.4	71.4	0.0230	0.0343	0.408
CO_2-cas.	10.8	51.4	88.8	0.0305	0.1480	0.689

almost 2× faster than Gln preceded by leucine. Micellar structural features in CO_2-casein may bring amino acids such as glycine, serine or arginine closer to Asn and Gln to enhance or catalyze deamidation. Knowledge of exact micelle structure could verify involvement of these amino acids. However, models of tertiary or three-dimensional structure of casein is limited (Swaisgood, 1992).

To best model the deamidation kinetics, a rate equation that expressed exponential first-order dependency with respect to the overall amide content was proposed. Previous food protein deamidation studies had employed a first-order kinetic model (Zhang et al., 1993c; Metwalli and van Boekel, 1998) with good accuracy for reaction times up to 3h.

The first-order rate equation was

$$R_A = d(C_A)/dt = -k\,C_A^n \tag{1}$$

in which R_A is the rate at which amide is depleted due to the deamidation reaction, k is the reaction rate constant, n is the reaction order, equal to 1 in this case, and t is the reaction time. After integration

$$C_A = C_{Ao}\,\exp[(-k)(t)] \tag{2}$$

The accuracy of the fit of the curve from Eq (2) to the experimental data was evaluated by a residual sum of squares. Similar to Zhang et al. (1993a), and Metwalli and van Boekel (1998), this first-order model fit our data well at early reaction times ($r^2>0.97$). At longer reaction times, however, the model curve diverged from the experimental data. Although the long reaction time of 96h may not be commercially practical, the study enables a more accurate assessment of reaction kinetic parameters such as order and rate constants. In addition, the kinetic constants could be used for short time periods as well as any time beyond with equal confidence.

Consequently, a first-order model that described deamidation kinetics in terms of the concentration of each amide, Asn and Gln, instead of the total amide concentration was developed. The total amide content of casein was given as

$$C_A = C_{Asn} + C_{gln} \tag{3}$$

in which C_{Asn} and C_{Gln} were the concentrations of Asn and Gln, respectively. With first-order concentration dependency for each amide proposed,

$$d(C_{Asn})/dt = -k_{Asn}\,C_{asn} \tag{4}$$
$$d(C_{Gln})/dt = -k_{Gln}\,C_{gln} \tag{5}$$

k_{Asn} and k_{Gln} represented the reaction rate constants for the respective amides. Eqns. (4) and (5) were integrated,

$$C_{Asn} = (C_{Asn})_o\,\exp[(-k_{Asn})(t)] \tag{6}$$
$$C_{Gln} = (C_{Gln})_o\,\exp[(-k_{Gln})(t)] \tag{7}$$

$(C_{Asn})_o$ and $(C_{Gln})_o$ were the respective initial amide concentrations. Their values were assumed to be those encountered in casein of standard composition (Eigel et al., 1984), which was 31.4 and 68.6 %, respectively, of the total casein amide content, C_{Ao}.

To evaluate the model, Eq (6) and (7) were substituted into Eq (3)

$$C_A = (C_{Asn})_o\,\exp[(-k_{Asn})(t)] + (C_{Gln})_o\,\exp[(-k_{Gln})(t)] \tag{8}$$

As mentioned, C_A was measured, and $(C_{Asn})_o$ and $(C_{Gln})_o$ were based on the measured C_{Ao} and on casein of standard composition. Thus, the set of points, C_A vs t, was fitted to the data by regression modeling. A representative plot of the experimental data points vs. model curve was constructed (Fig. 1). This first-order model displayed good fit ($r^2 > 0.97$) for all caseins throughout the reaction.

The reaction rate constants of the individual amides were determined from parameters produced by the regression modeling (Table 3). As expected, Asn values were much higher than those for Gln. This was consistent with the size of the cyclic imide ring intermediate formed during deamidation at basic pH. The Asn ring contains one less methyl group than the Gln ring. The smaller Asn ring decreases steric hindrance between peptide nitrogen and side-chain carbonyls which could lead to faster intermediate ring formation and subsequent deamidation for Asn as compared to Gln (Geiger and Clarke, 1987; Stephenson and Clarke, 1989; Tyler-Cross and Schirch, 1991; Wright, 1991b).

The temperature dependence of the reaction rate constants was examined by constructing plots of reaction rate constants at the three experimental temperatures (Figs. 2 and 3). Resulting regression equations should be linear and of the familiar Arrhenius form

$$\ln k_i = -(E_i/R)(1/T) + \ln A_i \tag{9}$$

where k_i is the reaction rate constant of either Asn or Gln, E_i is its

activation energy, R is the universal gas constant, T is the temperature expressed in (°K, and A_i is the frequency factor. The Arrhenius plot for Gln (Fig. 3) showed firm linearity, while the plot for Asn (Fig. 2) showed a change in slope. The decrease in slope from the 85–100°C range to 100–115°C range for all caseins specifically indicated that even though deamidation reaction rate increased with temperature, this rate increase was retarded as temperature increased. The change in slope suggests a change in the mechanism of deamidation with respect to temperature for Asn. Metwalli and van Boekel (1998) showed no such change in slope for deamidation of sodium caseinate; however their temperature range was 110–145°C. Zhang et al. (1993b) showed a change in slope with respect to soy protein deamidation over a range of 100–130°C.

Activation energies were determined from the slopes of these plots (Table 4). In the case of Asn, two activation energies were determined, corresponding to the change in slope. The Arrhenius equation could be used to calculate reaction rate constants and consequently calculate the extent of deamidation at various temperatures and times using Eq (8). For example, we generally find good agreement (within 0.9 to 10.5 %) between the experimental extent of deamidation of sodium caseinate reported by Metwalli and van Boekel (1998) at 140°C and the extent predicted from our reaction rate constants for sodium casein-

Table 4—Deamidation activation energies (kJ/mol) of individual casein amides

	Ca-cas.	Na-cas.	CO_2-cas.
Asn (85-100°C)	84.6	97.2	113
Asn (100-115°C)	25.1	52.0	43.9
Gln	268	199	120

ate at 140°C (Arrhenius parameters for higher temperatures of 100–115°C were used to calculate Asn rate constant).

CONCLUSIONS

THE EXTENT OF THERMAL DEAMIDATION OF CO_2-CASEIN AT 85, 100, and 115°C was greater than that of commercial sodium and calcium caseinates at the higher temperatures of 100 and 115°C after 32 and 24h, respectively. Structural differences between CO_2-casein and the caseinates may potentially be a factor in the different extents of deamidation. During precipitation from milk, CO_2-casein may retain some micellar structure while the caseinates do not. The kinetics of deamidation appeared to follow a first-order dependence with respect to each of the amides, Asn and Gln. Corresponding Arrhenius expressions could be used to calculate reaction rate constants at different temperatures. Subsequently, using the first-order rate equation, the extent of deamidation could be calculated for any particular time.

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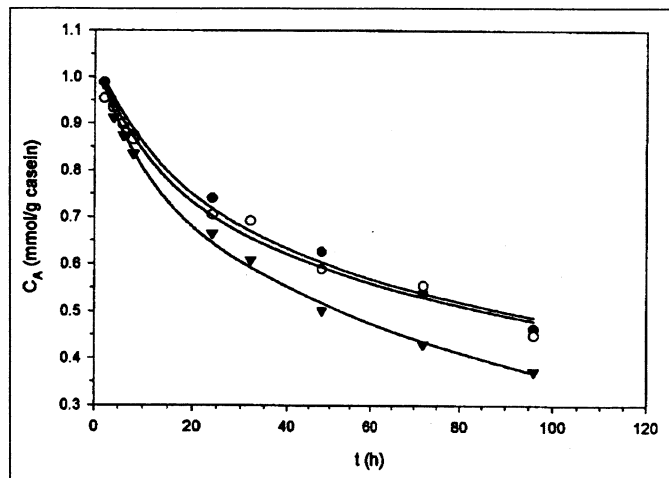
Fig. 1—Deamidation of caseins at 115°C. Solid lines represent model curves. Experimental data points: (●) calcium caseinate; (○) sodium caseinate; (▼) CO_2-casein.
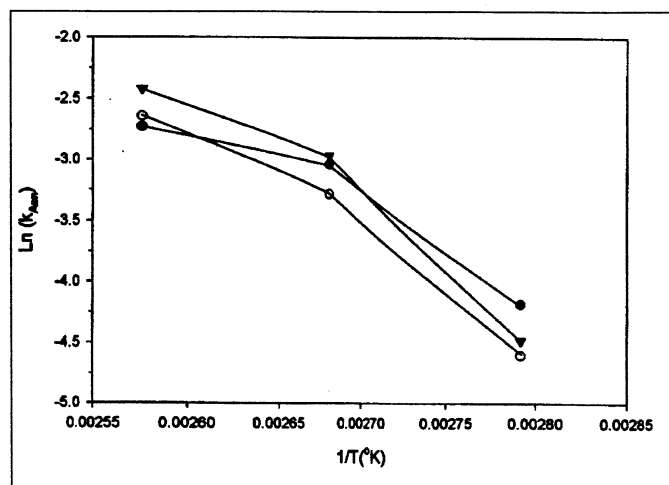

Fig. 2—Arrhenius plot for asparagine deamidation of casein. (●) calcium caseinate; (○) sodium caseinate; (▼) CO_2-casein.
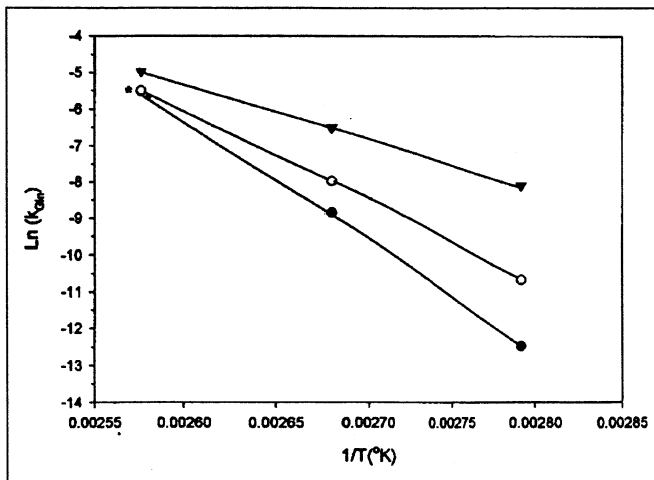

Fig. 3—Arrhenius plot for glutamine deamidation of casein. (●) calcium caseinate; (○) sodium caseinate; (▼) CO_2-casein. *Data point for calcium caseinate behind data point for sodium caseinate.

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